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Novel mutations in *PANK2* and *PLA2G6* Genes in Patients with Neurodegenerative Disorders: two case reports

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Abstract

Background: Neurodegeneration with brain iron accumulation (NBIA) is a genetically heterogeneous group of disorders associated with progressive impairment of movement, vision, and cognition. The disease is initially diagnosed on the basis of brain magnetic resonance imaging findings which indicate an abnormal brain iron accumulation in the basal ganglia. However, the diagnosis of specific types should be based on both clinical findings and molecular genetic testing for genes associated with different types of NBIA, including *PANK2, PLA2G6, C19orf12, FA2H, ATP13A2, WDR45, COASY, FTL, CP*, and *DCAF17.* The purpose of this study was to investigate disease-causing mutations in two patients with distinct NBIA disorders.

Case presentation: Whole Exome sequencing using Next Generation Illumina Sequencing was used to enrich all exons of protein-coding genes as well as some important other genomic regions in these two affected patients. A deleterious homozygous four-nucleotide deletion causing frameshift deletion in *PANK2* gene (c.1426_1429delATGA**,** p.M476fs) was identified in an 8 years old girl with dystonia, bone fracture, muscle rigidity, abnormal movement, lack of coordination and chorea. Also our study revealed a novel missense mutation in *PLA2G6* gene (c.G3T:p.M1I) in a half year-old boy with muscle weakness and neurodevelopmental regression

(speech, motor and cognition). The identified novel mutations were also confirmed by Sanger sequencing in the proband and their parents.

Conclusions: current study uncovered two rare pathogenic mutations in *PANK2* and *PLA2G6* genes in patients with NBIA disorder and such studies may help to conduct genetic counselling and prenatal diagnosis more accurately for individuals at the high risk of these types of disorders.

Keywords: *PLA2G6*, PKAN; NBIA; *PANK2;* Case report

Background

Neurodegeneration with brain iron accumulation (NBIA) is etiologically and clinically a heterogeneous group of inherited neurologic disorders characterized by basal ganglia iron deposition, mainly in the globus pallidus and/or substantia nigra. The hallmark of NBIA include dystonia, dysarthria, spasticity, and Parkinsonism [1-4]. However, apart from these neurological manifestations and Neuropathologic findings such as axonal spheroids, other abnormalities like retinal degeneration and optic atrophy are common in patients with NBIA [3, 4]. Up to now, the genetic basis of ten types of NBIA has been established which include Aceruloplasminemia [5], Beta-propeller protein-associated neurodegeneration [6], COASY protein-associated neurodegeneration [7, 8], Fatty acid hydroxylase-associated neurodegeneration [9, 10], Kufor-Rakeb syndrome [11], mitochondrial membrane protein-associated neurodegeneration [12, 13], Neuroferritinopathy [14, 15], *PLA2G6*-associated neurodegeneration (PLAN) [16-18], Pantothenate kinase-associated neurodegeneration (PKAN) [19], and Woodhouse-Sakati syndrome [20, 21]. It has been described that the major percentage of NBIA is attributed to autosomal recessive mutations in Pantothenate Kinase 2 (*PANK2*) gene [22], which is resulted in PKAN [19], and Phospholipase A2 Group VI (*PLA2G6*) gene, leading to PLAN [18, 23].

PKAN is divided into two types which include classic PKAN, with early onset in the first decade of life and rapid progression, and atypical PKAN with rare, later onset and slower progression [22]. Children

with PKAN typically have gait difficulties approximately at the age of three and at later life they usually show progressive dystonia, rigidity, dysarthria, and spasticity. However, patients with later-onset PKAN present speech difficulty and psychiatric symptoms [24, 25]. It worth noting that in individuals with PKAN, Magnetic Resonance Imaging (MRI) is characterized by ‘‘eye-of-the-tiger’’ sign, T2-hypointensity of the globus pallidus with a central hyperintensity, corresponding to excessive brain iron accumulation [26] and predicting a disease causing mutation in *PANK2* gene [27]. However, mutation detection is a gold standard to confirm diagnosis in a patient even if the radiologic findings show the typical eye-of-the-tiger sign since there is no a strong correlation between this sign and *PANK2* mutation. Another main form of NBIA is PLAN which is caused by mutation in *PLA2G6* gene. PLAN is characterized by three phenotypes, including infantile neuroaxonal dystrophy (INAD), atypical neuroaxonal dystrophy (NAD), and *PLA2G6*-related dystonia-parkinsonism [28, 29]. INAD phenotype which is occurred between ages 6 months and 3 years is usually manifested with developmental regression, progressive psychomotor delay, initial hypotonia and progressive spastic tetraparesis. Regarding the atypical NAD which is commonly observed with slower progression, dystonia, spastic tetraparesis, speech delay and diminished social interactions, is presented later in childhood [30-32]. By contrast, the third phenotype, *PLA2G6*-related dystonia-parkinsonism, is manifested in late adolescence/early adulthood with marked cognitive decline, pyramidal tract signs and eye movement abnormalities. It should be noted that in brain MRI imaging, the hallmark features of both INAD and atypical NAD is recognized as cerebellar atrophy and optic atrophy and in more cases, brain iron accumulation usually in the globus pallidus is detected [29, 33].

By the fact that up to now various genes (*PANK2*, *PLA2G6*, *C19orf12*, *FA2H*, *ATP13A2*, *WDR45*, *COASY*, *FTL*, *CP*, and *DCAF17* [34]*)* have been shown to be associated with different types of NBIA and other neurodegenerative disorders, the aim of this study was to investigate disease-causing mutations using NGS method in our two patients with neuromuscular and neurodegenerative disorders.

Case Presentation:

Here we report two Iranian and Afghani patients born in consanguineous families affected by NBIA. The diagnoses had been made on the basis of the clinical findings of a progressive movement disorder.

Family I, Patient I: An 8 years old Iranian girl was admitted to Namazi Hospital (Shiraz, Iran) in 2015 with clinical diagnosis of dystonia who was apparently normal before the age of 4. She developed bone fracture, muscle rigidity, abnormal movement, lack of coordination, chorea, and dystonia with seizure attacks. She was intellectually normal but she had speech problem due to medications she was taking which were Sirdalud (Tizanidine), Gabax, trihexidine and NA Valporate.

Multiplanar multisequential MRI images through the brain with usual protocol were taken which demonstrated normal signal intensity of both cerebral hemispheres with no sign of mass or hemorrhage or ischemic infarction. No hydrocephalus or shift of midline structure was found. Posterior fossa structures including cerebral hemispheres showed normal signal intensity without any mass or hemorrhage or ischemic infarction. 7the-8the nerve root complexes appeared normal and pituitary gland was also normal with no sign of gross mass. No extra-axial mass or hematoma or fluid collection was observed. It is worth noting that generalized cortical atrophy was considerable which was more than that of expected for the patient's age. Mucosal thickening was noted at both ethmoidal maxillary sinuses due to sinusitis. Mild inflammatory change at right mastoid air cells and the ''eye-of-the-tiger'' sign in MRI imaging was remarkable (figure 1). But, M.R.I of the cervical spine without contrast showed normal features.

Paraclinical examinations were also requested which showed increased level of alkaline phosphatase (ALP) (191 U/L) and creatine phosphokinase (CPK) (456 U/L).

Family II, Patient II: One and half year-old Afghani boy with muscle weakness at the onset of disease (a case of neuromuscular disease) was addmited to comprehensive children's development in Emam Reza Hospital (Shiraz, Iran) in 2014. He has not been on any treatment until now. Diagnostic evaluations were brain MRI and abdominal and pelvic ultrasonography. There was no intellectual impairment and no hepatosplenomegaly at that age. At the age of two, he showed neurodevelopmental regression (speech, motor and cognition) and floppy infant (hypotonia) but there was no deep tendon

reflexes (DTR) and no seizure. The ultrasonography showed normal features but in MRI imaging only a minimal change of periventricular white mater was observed which could be due to mild delayed myelination. Two of his sisters died with similar phenotype at the age of six and four years.

Comprehensive laboratory examinations were also requested, including hematology, biochemistry, hormone, and urine analaysis. The positive and abnormal findings for this patient were the decreased level of hemoglobin (Hb) (11.8 g/dL), hematocrit (HCT) (34.5 %), mean corpuscular volume (MCV) (68.73 fL), mean corpuscular hemoglobin (MCH) (23.51 pg), and increased level of CPK (1124 U/L), lactate dehydrogenase (LDH) (542 µ/L), and aspartate aminotransferase (AST, SGOT) (64 U/L) enzymes.

Genetic tests for SMA and DMD diseases showed negative results and therefore whole exom sequencing was suggested to the family.

Next Generation Sequencing:

Whole Exome Sequencing was utilized for amplification and sequencing of all exons of protein-coding genes as well as some important other genomic regions. The DNA samples were sequenced, using Illumina HiSeq2000 machine and standard Illumina protocol for pair-end 99-nucleotide sequencing. Detail of sample alignment is listed below in Table 1. Briefly, next generation sequencing was performed to sequence close to 100 million reads on Illumina HiSeq2000 Sequencer. In general, test platform examined >95% of the targeted regions with sensitivity of above 99%. In this test, point mutations and micro-insertion/deletions and duplication (<20bp) can be simultaneously detected. Bioinformatics analysis of the sequencing results was performed using BWA aligner [35], GATK [36] and annovar [37] open access software as well as public databases and standard bioinformatics software.

Sanger sequencing and segregation studies:

Whole blood samples were collected in EDTA tubes from family members of the probands and then genomic DNA was extracted from the peripheral blood lymphocytes by QIAamp DNA Blood Mini Kit (Germany) according to the manufacturer's instructions. After that, the genomic DNA concentration was measured by NanoDrop (ND1000, USA) and stored at −20 °C until use.

To confirm the novel identified mutations, PCR was performed for the probands and their parents (PCR condition are given in table 2) and amplified DNA was then subjected to Sanger Sequencing using both forward and reverse primers according to ABI BigDye Terminator Cycle Sequencing Kit (Applied Biosystems®, USA). Sanger sequencing data was analyzed using NCBI BLAST and CodonCode Aligner software. Multiple sequence alignment analysis extracted from Polyphen website was also used to compare the amino acid sequence of human PANK2 and PLA2G6 protein with corresponding proteins across all Kingdoms. Following bioinformatics software and websites were also used to identify the features of PANK2 and PLA2G6 and the consequences of mutations in the given position of the proteins: Polyphen, Mutation Taster, SIFT, and DISOPRED3 (Intrinsic disorder predictor).

Whole exome sequencing utilizing next generation sequencing was performed on DNA samples from patients, on an Illumina platform. Sequences text files were aligned using BWA aligner tool and variants were identified using GATK and annotated utilizing annovar software. In family I, a deleterious novel homozygous four-nucleotide deletion causing frameshift deletion gene (NM_153638: exon 5, c.1426_1429delATGA, p.M476fs) was identified in *PANK2* gene. Mutations and small deletions in *PANK2* gene have been reported in patients with NBIA1(OMIM: 234200). The disease also called PKAN and apparently causes dystonia in affected individuals. Regarding the family II, a deleterious novel homozygous missense mutation was found in *PLA2G6* gene (NM_001004426: exon 2: c.G3T: p.M1I). These identified mutations were not reported before and therefore, classified as variation of unknown significance (VUS). Using Sanger sequencing, these mutations were also confirmed in probands and theirs parents, showing their autosomal recessive inheritance (figure 1A and 2A).

Novel Mutation in *PANK2* causing Dystonia:

Pantothenate kinase which is a ubiquitous and major cofactor in all organisms plays a central role as an essential regulatory enzyme in the metabolism of carboxylic acids, such as coenzyme A (CoA). It catalyzes the first and rate limiting step in the universal five step CoA biosynthesis pathway and its activity is regulated primarily through feedback inhibition by acyl CoA species [38-40]. Up to now, three distinct types of pantothenate kinase enzymes have been identified which include type I (a

prokaryotic PanK that predominates in eubacteria), type II (mainly in eukaryotic organisms), and type III (with a wider phylogenic distribution) [41].

PANK2 which appears to be the only mitochondria-targeted human PanK is involved in a myriad of metabolic reactions, including metabolism of water-soluble vitamins (such as B5) and cofactors [42]. This gene is located on chromosome 20 (20p13) consisting of 7 exons [19] and different isoforms are generated by alternative PANK2 mRNA splicing with the use of alternate first exons. But as reported in literature only two PanK2 protein isoforms are proteolytically produced to form a mitochondrially localized, mature PanK2 [43]. Mutations in these isoforms are associated with HARP syndrome and PKAN, formerly Hallervorden-Spatz syndrome.

Approximately 100 mutations in *PANK2* have been found in affected individuals with PKAN [19, 44-46]. The most common *PANK2* mutations are G411R and T418M accounted for one-third of the disease alleles [19]. Usually patients with the severe early-onset form of the disorder have *PANK2* mutations that is resulted in the complete absence of functional PANK2 [47]. But the disease in cases affected by the later-onset form is typically resulted from changes of single amino acids in the enzyme producing a protein retaining some functional properties [22, 48]. So the residual activity of PANK2 in mitochondria determines the age of disease onset and is proposed to be the best indicator of clinical findings [48]. It is well recognized that PKAN symptoms (classic PKAN) are usually manifested in early childhood while atypical PKAN is referred to the condition presented in teenage life. According to our data, onset in our PANK2-positive patient was 4 years and, therefore this case would be classified as ‘‘classic PKAN. This patient was found to be homozygous for *PANK2* deletion mutation at position c.1426_1429delATGA, p.M476fs in exon5. This mutation has not previously been reported and may be associated with early onset and rapid progression disease. Following evidences confirm that this mutation results in PKAN:

1- Whole exome sequencing using next generation sequencing only revealed this mutation to be the cause of PANK in the patient. **2-** As shown in figure 1A, using Sanger sequencing, the mutation was confirmed in the proband and the inheritance pattern based on heterozygote mutation identified in her parents must be an autosomal recessive mode. **3-**This four-nucleotide deletion (c.1426_1429delATGA) causes frameshift after codon 476 in PANK2 protein, leading to the premature translation termination

and making it highly likely to contribute to the observed phenotype in the patient. 4- Despite the mutation is in the 3' end of the open reading frame of this protein, it is predicted to produce a completely nonfunctional truncated polypeptide since one of the reported transcript for this gene (ENST00000336066.7, V9GYZ0) with the absence of all amino acids after position 279 is resulted in nonsense mediated decay (figure 1B). Also, using Clustal W Multiple Sequence Alignment (figure 1C), it can be seen that after codon 191 all amino acid are included in all functional isoforms of PANK2, representing the vital presence of these codon in the protein. **5-** This mutation is close to similar mutations in *PANK2* gene that has been reported to cause NBIA in the basal ganglia of the brain. **6-** According to Mutation Taster online software, this variation predicted to be a disease causing variant **7-** The comparative amino acids alignment of PANK2 protein across all Kingdoms was also performed by using multiple sequence alignment analysis extracted from Polyphen website and as shown in figure 1D, residues in this region is highly conserved during evolution. As a result, these evidence can prove that this deletion mutation in *PANK2* gene is extremely pathogenic in patients with PANK.

Novel Mutation in *PLA2G6* causing PLAN:

PLA2G6, Calcium-Independent Phospholipase A2 Group VI, which catalyzes the release of fatty acids from phospholipids may have a role in normal phospholipid remodeling, vasopressin-induced arachidonic acid release, leukotriene and prostaglandin production, fas-mediated apoptosis, and transmembrane ion flux in glucose-stimulated B-cells [49]. *PLA2G6* located on 22q13.1 consists of 17 exons which is subjected to transcription of several encoding isoforms, however, until now, only the features of its three full-length transcripts have been reported and abnormal function of this PLA2 group VI enzyme may impair the integrity of cell membrane, leading to several neurodegenerative disorders [28, 29].

It has been found that various mutations in *PLA2G6* are associated with parkinson disease 14 [50], autosomal recessive, INAD1[28, 32], Neurodegeneration with brain iron accumulation 2A (NBIA2A) and 2B (NBIA2B) [28, 31].

PARK14 [MIM:612953] which is a progressive neurodegenerative disorder with an adult-onset is characterized by parkinsonism, dystonia, severe cognitive decline, cerebral and cerebellar atrophy and absent iron in the basal ganglia on magnetic resonance imaging [50]. Regarding the NBIA2A [MIM: 256600], it is a neurodegenerative disease characterized by the unique pathological feature of NAD, including axonal swelling and spheroid bodies in the central nervous system. The typical symptoms of the disease is started in the first 2 years of life and finally is led to the death around the age of 10 years. In relation to the NBIA2B [MIM: 610217], it is a neurodegenerative disorder with iron accumulation in the brain, primarily in the basal ganglia, and characterized by progressive extrapyramidal dysfunction leading to rigidity, dysarthria, sensorimotor impairment and dystonia [28, 31]. Concerning the INAD, it is a rare autosomal recessive neurodegenerative disorder with axonal swell and high brain iron resulting to intellectual disability and movement problems. At least 50 mutations in the *PLA2G6* gene have been identified in people with INAD [28, 32].

In our study a novel homozygous mutation in *PLA2G6* gene (c.G3T:p.M1I) was identified in an Afghani patient with INAD phenotype (due to age of disease onset, at the age of 1 and half year, and manifestations of developmental regression and progressive psychomotor delay) and following evidences can prove that this mutation results in PLAN:

1- c.G3T mutation is caused the first codon ,ATG, to be shifted, leading to abnormal protein and making it highly likely to contribute to the observed phenotype in the patient. **2-** This mutation is close to similar mutation in first codon of *PLA2G6* gene (Met1Val) [32] that has reported to lead to NBIA (INAD1 form) **3-** Whole exome sequencing only identified this mutation to be the main cause of PLAN in the patient. **4-** As shown in figure 2A, using Sanger sequencing, the mutation was confirmed in the proband and on the basis of identified heterozygote mutation in his parents, the inheritance pattern must be an autosomal recessive mode. **5-** Mutation Taster, SIFT, and Polyphen online software predicted that this variation will be damaging **6-** As can be seen in figure 2B, the comparative amino acids alignment of PLA2G6 protein across all Kingdoms using multiple sequence alignment analysis extracted from Polyphen website showed that this residue is highly conserved during evolution. **7-** Intrinsic disorder profile for PLA2G6 predicted by DISOPRED3 revealed that amino acids in some region of protein

including the first amino acids are considered disordered when the dark line is above the grey dashed line (figure 2C). This amino acids are also involved in protein binding and, therefore they are very important in its functional state (figure 2C). As a result, this mutation in *PLA2G6* gene is extremely pathogenic in patient with PLAN.

To understand the pathomechanism of PLAN and PKAN characterized by degenerative changes of neuronal tissues, it is essential to identify the *PANK2* and *PLA2G6* mutations. It has been shown that different mutations in *PLA2G6* and *PANK2* are caused distinct neurological disorders with a heterogeneity of phenotypes and a variable age of disease onset which may be due to disrupted interactions between these proteins and their possible predicted partners in a complex protein network. Up to now, no drugs have been used to treat the disorder, and the initial step in drug discovery research is finding out essential proteins or drug targets for a biological process. To identify that possible interactions between these two proteins and other partners may play important roles in pathogenesis of NBIA and other neurodegenerative, we used STRING software (Search Tool for the Retrieval of Interacting Genes/Proteins: string.embl.de/) and as shown in figure 3 and 4, several predicted functional partners interacting PLA2G6 and PANK2 were identified. It worth noting that these two protein is also predicted to have an interaction with each other and therefore they may have roles in the same complex protein network involved in Iron metabolism. Understanding the exact mechanism of these predicted protein and pathways may shed light into therapeutic strategies for NBIA and related neurodegenerative disorders with the use of these proteins (through their up or down regulation) or any known drugs.

Conclusions

Two rare pathogenic mutations in *PANK2* and *PLA2G6* genes were identified in our patients with neuromuscular and NBIA disorders and such studies may help to conduct genetic counselling and prenatal diagnosis more accurately for individuals at the high risk of these types of disorders.

List of abbreviations:

ALP; alkaline phosphatase

AST; aspartate aminotransferase

CoA; coenzyme A

CPK; creatine phosphokinase

DTR; deep tendon reflexes

Hb; hemoglobin

HCT; hematocrit

INAD; infantile neuroaxonal dystrophy

LDH; lactate dehydrogenase

MCH; mean corpuscular hemoglobin

MCV; mean corpuscular volume

MRI; magnetic resonance imaging

NAD; neuroaxonal dystrophy

NBIA; neurodegeneration with brain iron accumulation

PKAN; pantothenate kinase-associated neurodegeneration

PLAN; PLA2G6-associated neurodegeneration

S.O.L; space-occupying lesion

Declarations

Ethics approval and consent to participate

Ethic committee at Shiraz University of Medical Sciences, Comprehensive Genetic center has approved the study and parents of affected individual has signed written consent indicating their voluntary contribution to the current study. A copy of the consent is available for review by the Editor of this journal.

Consent for publication

Not applicable

Availability of data and materials

All data including NGS sequencing raw and analyzed data and sanger sequencing files will be provided by corresponding author to interested scientist upon request. The identified mutation will be uploaded into HGMC database as well as ClinVar website.

Competing interests

The authors declare that there are no financial and non-financial competing interests.

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Authors' contributions

Dr.M.A.Faghihi conceived and designed the study, collected, assembled, interpreted NGS data and wrote the manuscript. Dr.M.Fardaei interpreted Sanger sequencing results of *PANK2* and *PLA2G6* genes and provide some funds. H.Dastssoz wrote the manuscript, designed *PANK2* primer, performed experiment, and interpreted Sanger sequencing results and bioinformatics analysis of *PANK2* and *PLA2G6* genes. Dr.H.Nemati clinically evaluated the patients and edited the manuscript. H.Firozi designed *PLA2G6* primer and collected samples from family 2.

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References

1. Dexter DT, Carayon A, Javoy-Agid F, Agid Y, Wells FR, Daniel SE, Lees AJ, Jenner P, Marsden CD: **Alterations in the levels of iron, ferritin and other trace metals in Parkinson's disease and other neurodegenerative diseases affecting the basal ganglia**. *Brain : a journal of neurology* 1991, **114 (Pt 4)**:1953-1975.
2. Gregory A, Hayflick SJ: **Genetics of neurodegeneration with brain iron accumulation**. *Current neurology and neuroscience reports* 2011, **11**(3):254-261.
3. Kruer MC, Paisan-Ruiz C, Boddaert N, Yoon MY, Hama H, Gregory A, Malandrini A, Woltjer RL, Munnich A, Gobin S *et al*: **Defective FA2H leads to a novel form of neurodegeneration with brain iron accumulation (NBIA)**. *Annals of neurology* 2010, **68**(5):611-618.
4. Dusek P, Jankovic J, Le W: **Iron dysregulation in movement disorders**. *Neurobiology of disease* 2012, **46**(1):1-18.
5. Miyajima H, Takahashi Y, Kono S: **Aceruloplasminemia, an inherited disorder of iron metabolism**. *Biometals : an international journal on the role of metal ions in biology, biochemistry, and medicine* 2003, **16**(1):205-213.
6. Hayflick SJ, Kruer MC, Gregory A, Haack TB, Kurian MA, Houlden HH, Anderson J, Boddaert N, Sanford L, Harik SI *et al*: **beta-Propeller protein-associated neurodegeneration: a new X-linked dominant disorder with brain iron accumulation**. *Brain : a journal of neurology* 2013, **136**(Pt 6):1708-1717.
7. Annesi G, Gagliardi M, Iannello G, Quattrone A, Iannello G, Quattrone A: **Mutational analysis of COASY in an Italian patient with NBIA**. *Parkinsonism & related disorders* 2016, **28**:150-151.
8. Dusi S, Valletta L, Haack TB, Tsuchiya Y, Venco P, Pasqualato S, Goffrini P, Tigano M, Demchenko N, Wieland T *et al*: **Exome sequence reveals mutations in CoA synthase as a cause of neurodegeneration with brain iron accumulation**. *American journal of human genetics* 2014, **94**(1):11-22.
9. Kruer MC, Gregory A, Hayflick SJ: **Fatty Acid Hydroxylase-Associated Neurodegeneration**. In: *GeneReviews(R).* Edited by Pagon RA, Adam MP, Ardinger HH, Wallace SE, Amemiya A, Bean LJH, Bird TD, Fong CT, Mefford HC, Smith RJH *et al*. Seattle (WA); 1993.
10. Pierson TM, Simeonov DR, Sincan M, Adams DA, Markello T, Golas G, Fuentes-Fajardo K, Hansen NF, Cherukuri PF, Cruz P *et al*: **Exome sequencing and SNP analysis detect novel compound heterozygosity in fatty acid hydroxylase-associated neurodegeneration**. *European journal of human genetics : EJHG* 2012, **20**(4):476-479.
11. Hampshire DJ, Roberts E, Crow Y, Bond J, Mubaidin A, Wriekat AL, Al-Din A, Woods CG: **Kufor-Rakeb syndrome, pallido-pyramidal degeneration with supranuclear upgaze paresis and dementia, maps to 1p36**. *Journal of medical genetics* 2001, **38**(10):680-682.
12. Gregory A, Hartig M, Prokisch H, Kmiec T, Hogarth P, Hayflick SJ: **Mitochondrial Membrane Protein-Associated Neurodegeneration**. In: *GeneReviews(R).* Edited by Pagon RA, Adam MP, Ardinger HH, Wallace SE, Amemiya A, Bean LJH, Bird TD, Fong CT, Mefford HC, Smith RJH *et al*. Seattle (WA); 1993.
13. Schulte EC, Claussen MC, Jochim A, Haack T, Hartig M, Hempel M, Prokisch H, Haun-Junger U, Winkelmann J, Hemmer B *et al*: **Mitochondrial membrane protein associated neurodegenration: a novel variant of neurodegeneration with brain iron accumulation**. *Movement disorders : official journal of the Movement Disorder Society* 2013, **28**(2):224-227.

14. Wills AJ, Sawle GV, Guilbert PR, Curtis AR: **Palatal tremor and cognitive decline in neuroferritinopathy**. *Journal of neurology, neurosurgery, and psychiatry* 2002, **73**(1):91-92.
15. Crompton DE, Chinnery PF, Fey C, Curtis AR, Morris CM, Kierstan J, Burt A, Young F, Coulthard A, Curtis A *et al*: **Neuroferritinopathy: a window on the role of iron in neurodegeneration**. *Blood cells, molecules & diseases* 2002, **29**(3):522-531.
16. Illingworth MA, Meyer E, Chong WK, Manzur AY, Carr LJ, Younis R, Hardy C, McDonald F, Childs AM, Stewart B *et al*: **PLA2G6-associated neurodegeneration (PLAN): further expansion of the clinical, radiological and mutation spectrum associated with infantile and atypical childhood-onset disease**. *Molecular genetics and metabolism* 2014, **112**(2):183-189.
17. Kurian MA, Hayflick SJ: **Pantothenate kinase-associated neurodegeneration (PKAN) and PLA2G6-associated neurodegeneration (PLAN): review of two major neurodegeneration with brain iron accumulation (NBIA) phenotypes**. *International review of neurobiology* 2013, **110**:49-71.
18. Gregory A, Kurian MA, Maher ER, Hogarth P, Hayflick SJ: **PLA2G6-Associated Neurodegeneration**. In: *GeneReviews(R).* Edited by Pagon RA, Adam MP, Ardinger HH, Wallace SE, Amemiya A, Bean LJH, Bird TD, Fong CT, Mefford HC, Smith RJH *et al*. Seattle (WA); 1993.
19. Zhou B, Westaway SK, Levinson B, Johnson MA, Gitschier J, Hayflick SJ: **A novel pantothenate kinase gene (PANK2) is defective in Hallervorden-Spatz syndrome**. *Nature genetics* 2001, **28**(4):345-349.
20. Bohlega SA, Alkuraya FS: **Woodhouse-Sakati Syndrome**. In: *GeneReviews(R).* Edited by Pagon RA, Adam MP, Ardinger HH, Wallace SE, Amemiya A, Bean LJH, Bird TD, Fong CT, Mefford HC, Smith RJH *et al*. Seattle (WA); 1993.
21. Medica I, Sepcic J, Peterlin B: **Woodhouse-Sakati syndrome: case report and symptoms review**. *Genetic counseling* 2007, **18**(2):227-231.
22. Hayflick SJ, Westaway SK, Levinson B, Zhou B, Johnson MA, Ching KH, Gitschier J: **Genetic, clinical, and radiographic delineation of Hallervorden-Spatz syndrome**. *N Engl J Med* 2003, **348**(1):33-40.
23. Gregory A, Westaway SK, Holm IE, Kotzbauer PT, Hogarth P, Sonek S, Coryell JC, Nguyen TM, Nardocci N, Zorzi G *et al*: **Neurodegeneration associated with genetic defects in phospholipase A(2)**. *Neurology* 2008, **71**(18):1402-1409.
24. Diaz N: **Late onset atypical pantothenate-kinase-associated neurodegeneration**. *Case reports in neurological medicine* 2013, **2013**:860201.
25. Pellecchia MT, Valente EM, Cif L, Salvi S, Albanese A, Scarano V, Bonuccelli U, Bentivoglio AR, D'Amico A, Marelli C *et al*: **The diverse phenotype and genotype of pantothenate kinase-associated neurodegeneration**. *Neurology* 2005, **64**(10):1810-1812.
26. McNeill A, Birchall D, Hayflick SJ, Gregory A, Schenk JF, Zimmerman EA, Shang H, Miyajima H, Chinnery PF: **T2* and FSE MRI distinguishes four subtypes of neurodegeneration with brain iron accumulation**. *Neurology* 2008, **70**(18):1614-1619.
27. Zolkipli Z, Dahmoush H, Saunders DE, Chong WK, Surtees R: **Pantothenate kinase 2 mutation with classic pantothenate-kinase-associated neurodegeneration without 'eye-of-the-tiger' sign on MRI in a pair of siblings**. *Pediatric radiology* 2006, **36**(8):884-886.
28. Morgan NV, Westaway SK, Morton JE, Gregory A, Gissen P, Sonek S, Cangul H, Coryell J, Canham N, Nardocci N *et al*: **PLA2G6, encoding a phospholipase A2, is mutated in neurodegenerative disorders with high brain iron**. *Nat Genet* 2006, **38**(7):752-754.
29. Paisan-Ruiz C, Bhatia KP, Li A, Hernandez D, Davis M, Wood NW, Hardy J, Houlden H, Singleton A, Schneider SA: **Characterization of PLA2G6 as a locus for dystonia-parkinsonism**. *Ann Neurol* 2009, **65**(1):19-23.
30. Sadeh M: **Neurodegeneration associated with genetic defects in phospholipase A2**. *Neurology* 2009, **73**(10):819.

31. Kurian MA, Morgan NV, MacPherson L, Foster K, Peake D, Gupta R, Philip SG, Hendriksz C, Morton JE, Kingston HM *et al*: **Phenotypic spectrum of neurodegeneration associated with mutations in the PLA2G6 gene (PLAN)**. *Neurology* 2008, **70**(18):1623-1629.
32. Wu Y, Jiang Y, Gao Z, Wang J, Yuan Y, Xiong H, Chang X, Bao X, Zhang Y, Xiao J *et al*: **Clinical study and PLA2G6 mutation screening analysis in Chinese patients with infantile neuroaxonal dystrophy**. *Eur J Neurol* 2009, **16**(2):240-245.
33. Sina F, Shojaee S, Elahi E, Paisan-Ruiz C: **R632W mutation in PLA2G6 segregates with dystonia-parkinsonism in a consanguineous Iranian family**. *European journal of neurology* 2009, **16**(1):101-104.
34. Arber CE, Li A, Houlden H, Wray S: **Review: Insights into molecular mechanisms of disease in neurodegeneration with brain iron accumulation: unifying theories**. *Neuropathology and applied neurobiology* 2016, **42**(3):220-241.
35. Li H, Durbin R: **Fast and accurate short read alignment with Burrows-Wheeler transform**. *Bioinformatics* 2009, **25**(14):1754-1760.
36. McKenna A, Hanna M, Banks E, Sivachenko A, Cibulskis K, Kernytsky A, Garimella K, Altshuler D, Gabriel S, Daly M *et al*: **The Genome Analysis Toolkit: a MapReduce framework for analyzing next-generation DNA sequencing data**. *Genome Res* 2010, **20**(9):1297-1303.
37. Wang K, Li M, Hakonarson H: **ANNOVAR: functional annotation of genetic variants from high-throughput sequencing data**. *Nucleic Acids Res* 2010, **38**(16):e164.
38. Brown GM: **The metabolism of pantothenic acid**. *J Biol Chem* 1959, **234**(2):370-378.
39. Wieland O: **[Vitamin function of pantothenic acid in animal cell metabolism]**. *Munch Med Wochenschr* 1959, **101**(12):501-510.
40. Leonardi R, Zhang YM, Rock CO, Jackowski S: **Coenzyme A: back in action**. *Prog Lipid Res* 2005, **44**(2-3):125-153.
41. Gerdes SY, Scholle MD, D'Souza M, Bernal A, Baev MV, Farrell M, Kurnasov OV, Daugherty MD, Mseeh F, Polanuyer BM *et al*: **From genetic footprinting to antimicrobial drug targets: examples in cofactor biosynthetic pathways**. *Journal of bacteriology* 2002, **184**(16):4555-4572.
42. Johnson MA, Kuo YM, Westaway SK, Parker SM, Ching KH, Gitschier J, Hayflick SJ: **Mitochondrial localization of human PANK2 and hypotheses of secondary iron accumulation in pantothenate kinase-associated neurodegeneration**. *Ann N Y Acad Sci* 2004, **1012**:282-298.
43. Zhang YM, Rock CO, Jackowski S: **Biochemical properties of human pantothenate kinase 2 isoforms and mutations linked to pantothenate kinase-associated neurodegeneration**. *J Biol Chem* 2006, **281**(1):107-114.
44. Hortnagel K, Prokisch H, Meitinger T: **An isoform of hPANK2, deficient in pantothenate kinase-associated neurodegeneration, localizes to mitochondria**. *Hum Mol Genet* 2003, **12**(3):321-327.
45. Houlden H, Lincoln S, Farrer M, Cleland PG, Hardy J, Orrell RW: **Compound heterozygous PANK2 mutations confirm HARP and Hallervorden-Spatz syndromes are allelic**. *Neurology* 2003, **61**(10):1423-1426.
46. Ching KH, Westaway SK, Gitschier J, Higgins JJ, Hayflick SJ: **HARP syndrome is allelic with pantothenate kinase-associated neurodegeneration**. *Neurology* 2002, **58**(11):1673-1674.
47. Hayflick SJ: **Pantothenate kinase-associated neurodegeneration (formerly Hallervorden-Spatz syndrome)**. *J Neurol Sci* 2003, **207**(1-2):106-107.
48. Hartig MB, Hortnagel K, Garavaglia B, Zorzi G, Kmiec T, Klopstock T, Rostasy K, Svetel M, Kostic VS, Schuelke M *et al*: **Genotypic and phenotypic spectrum of PANK2 mutations in patients with neurodegeneration with brain iron accumulation**. *Ann Neurol* 2006, **59**(2):248-256.

49. Tang J, Kriz RW, Wolfman N, Shaffer M, Seehra J, Jones SS: **A novel cytosolic calcium-independent phospholipase A2 contains eight ankyrin motifs**. *J Biol Chem* 1997, **272**(13):8567-8575.
50. Gui YX, Xu ZP, Wen L, Liu HM, Zhao JJ, Hu XY: **Four novel rare mutations of PLA2G6 in Chinese population with Parkinson's disease**. *Parkinsonism Relat Disord* 2013, **19**(1):21-26.

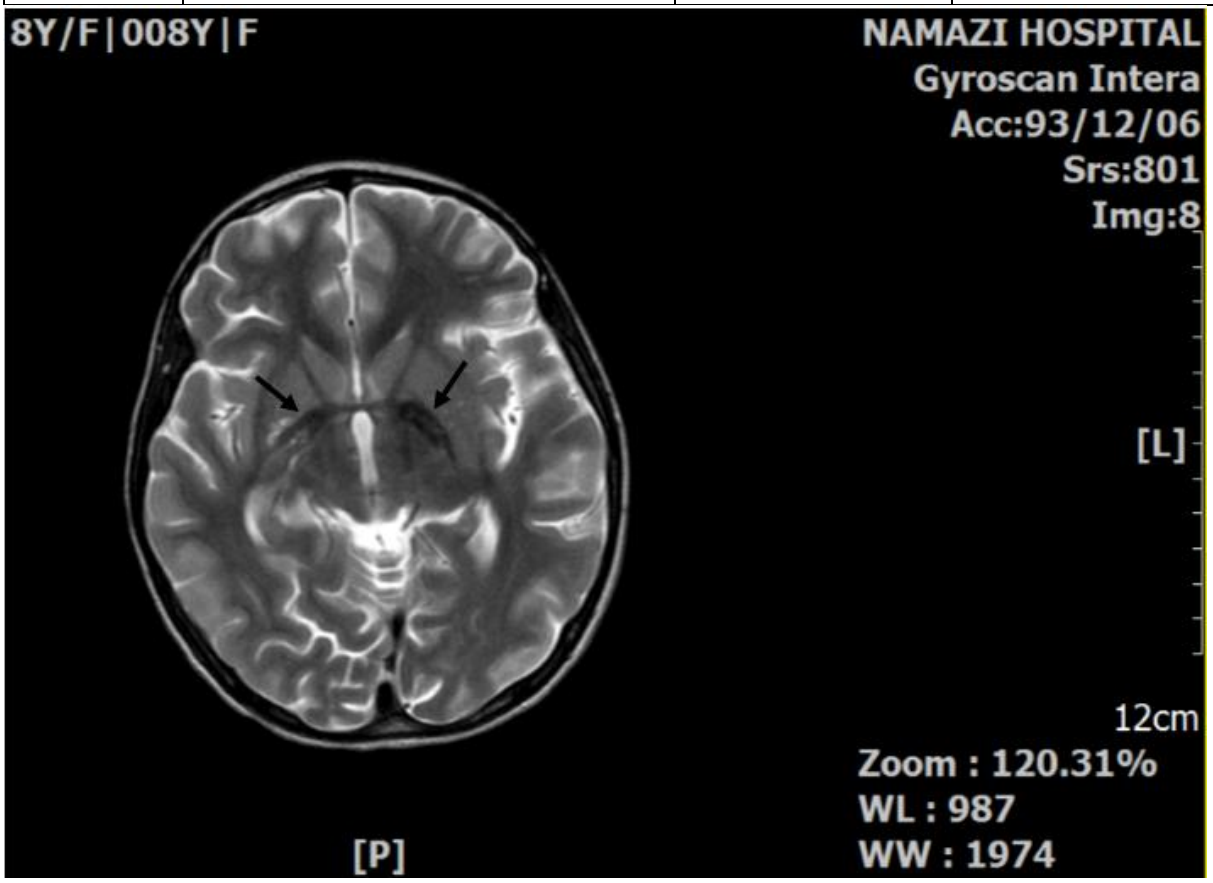
Table 1 Whole Exome Sequencing Detail of coverage and number of reads

Type	Value	Type	Value
Number of mapped reads	41,674,840	Percent reads on target	95.70%
Number of amplicons	293,903	Total assigned amplicon reads	39,882,524
Percent assigned amplicon reads	95.70%	Average reads per amplicon	136
Uniformity of amplicon coverage	86.30%	Amplicons with at least 100 reads	53.69%
Amplicons with at least 1 read	99.54%	Amplicons with at least 500 reads	0.70%
Amplicons with at least 20 reads	90.02%	Amplicons reading end-to-end	35.97%
Amplicons with no strand bias	85.64%	Total aligned base reads	7,342,243,527
Bases in target regions	57,742,646	Total base reads on target	6,979,820,754
Percent base reads on target	0.95	Uniformity of base coverage	0.85
Average base coverage depth	121	Target bases with no strand bias	78.31%
Target base coverage at 1x	99.18%	Target base coverage at 100x	47.95%
Target base coverage at 20x	87.91%	Target base coverage at 500x	0.62%
Percent end-to-end reads	58.98%	mapping rate	99.10%
AQ17	92.21%	AQ20	87.51%

493 **Table 2** Primer paires and PCR conditions to confirme novel mutations

Gene	Primer Sequence	PCR Product (bp)	PCR Program
PANK2	**Forward**: GTGTTGTCCTGGAACTGTCTG	**563**	95°C for 15 min, 35 cycles for: 95°C-30 sec, 60°C-30 sec, 72°C-30 sec, and final extension 72°C-7min
	Reverse: CCCACCCCAAATGACTACATTTA		
PLA2G6	**Forward**: GCCAATAAGACCTCCAATC	**515**	
	Reverse: GTCACTTTTACCTCCCACTC		

494



495 **Fig. 1** MRI featuers in patient with PKAN. T2-weighted brain MRI of the 8-year-old patient shows
496 bilateral symmetrical hypointensity in the globus pallidus with central hyperintensity, giving an eye-
497 of-the-tiger sign (arrows).

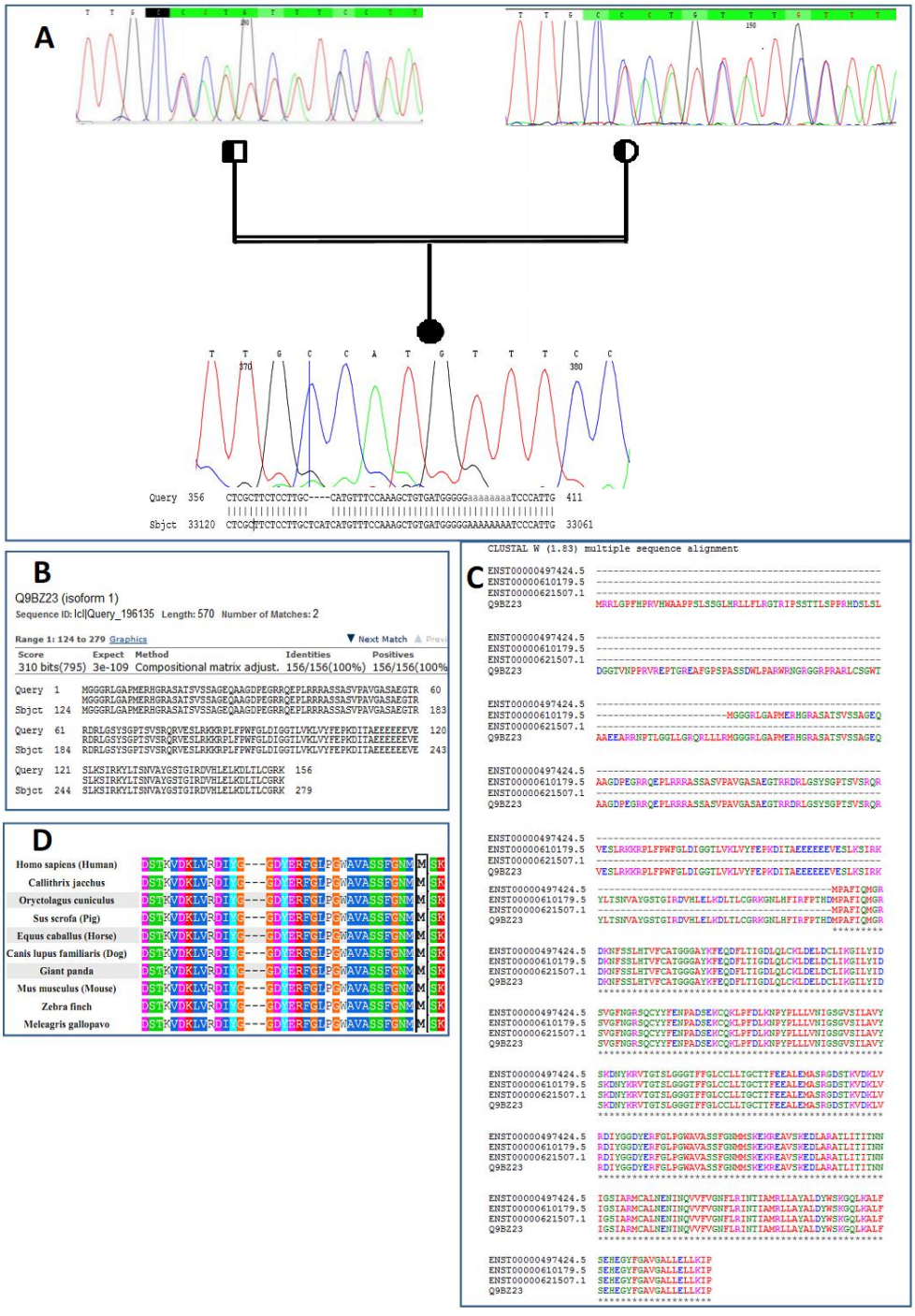


Fig. 2 Confirmation of new mutation in family I. **A).** Using Sanger sequencing, the inheritance mode of autosomal recessive was confirmed in this family on the basis of identified heterozygote mutation in parents and homozygote in the proband. **B).** PANK2 transcript leading to Nonsense mediated decay. **C)**. Multiple sequence alignment of all human encoding isoforms of PANK2 using Clustal W which shows the same conserved residues in these isoforms. **D).** Comparative amino acids alignment of PANK2 protein across all Kingdoms.

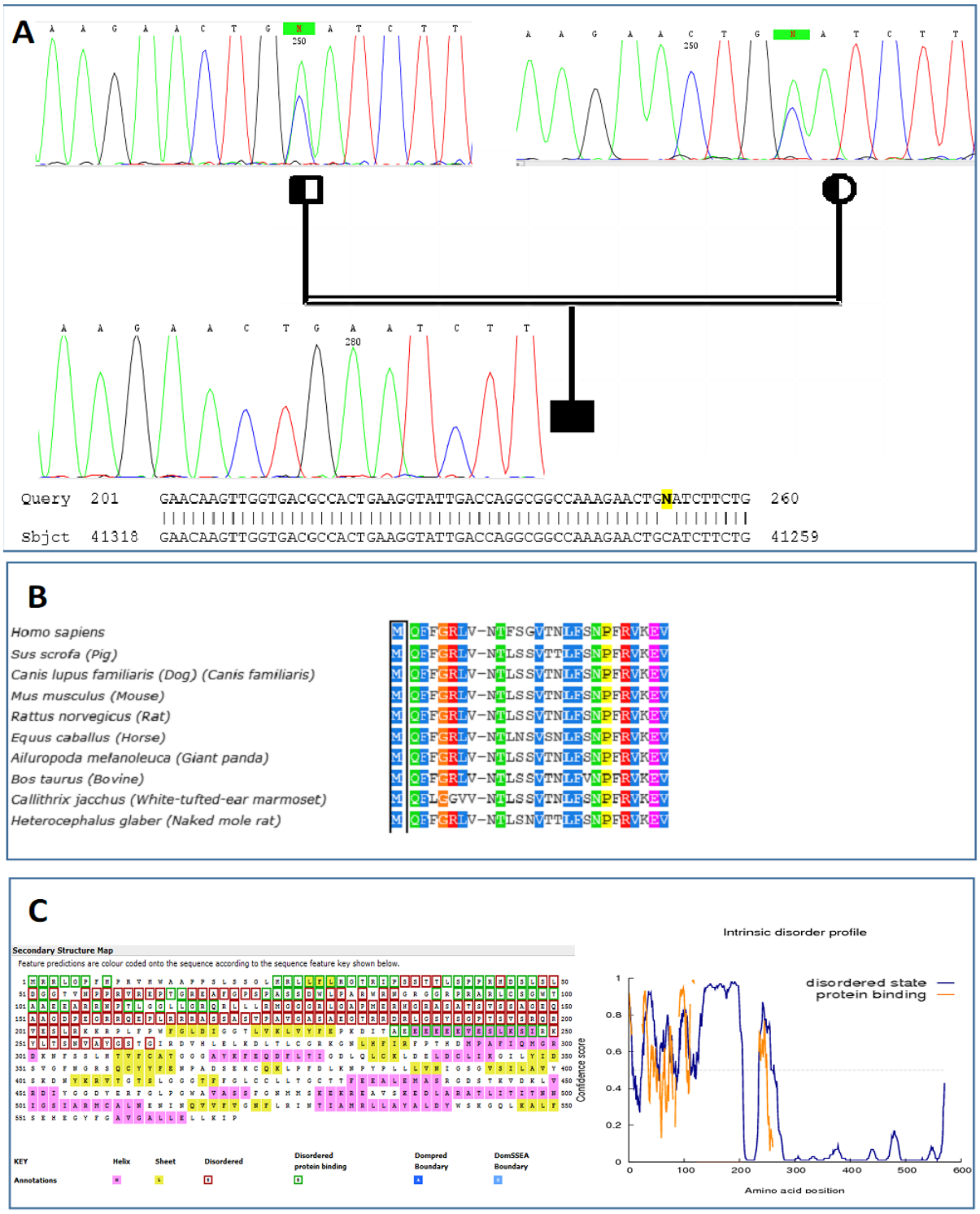
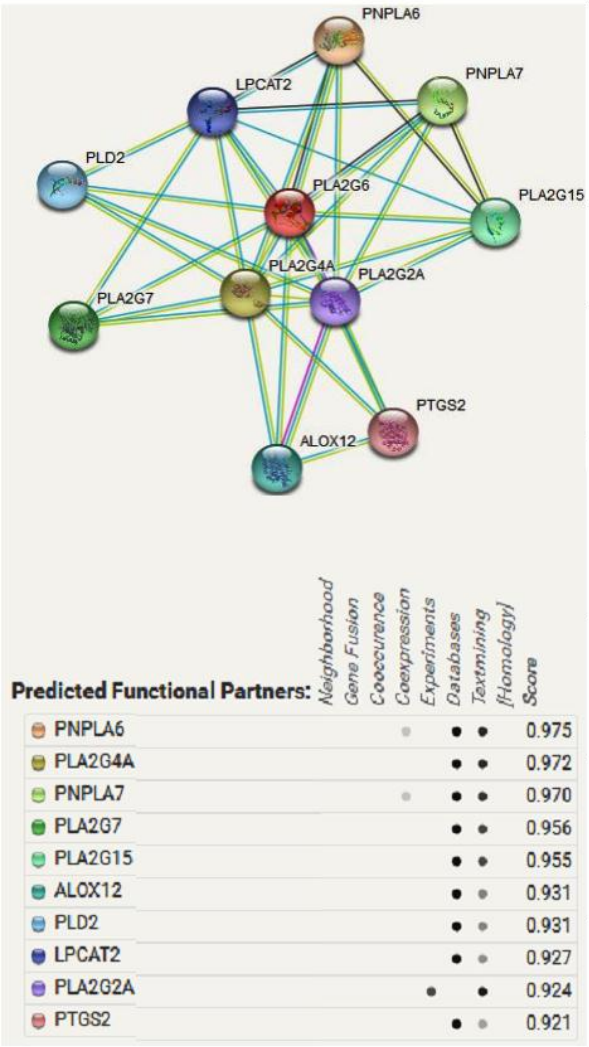


Fig. 3 Confirmation of novel mutation in family II. **A).** Confirmation of autosomal recessive pattern of *PLA2G6* mutation in the proband with PLAN disorder. **B).** Comparative amino acids alignment of PLA2G6 protein across all Kingdoms. **C).** Intrinsic disorder profile for PLA2G6 and its secondary structure map predicted by DISOPRED3. Amino acids in the input sequence are considered disordered when the dark line is above the grey dashed line, that is the confidence score is higher than 0.5.



PNPLA6 (Patatin Like Phospholipase Domain Containing 6) is a phospholipase that deacetylates intracellular phosphatidylcholine to produce glycerophosphocholine. It is thought to function in neurite outgrowth and process elongation during neuronal differentiation. The protein is anchored to the cytoplasmic face of the endoplasmic reticulum in both neurons and non-neuronal cells. The protein is the target for neurodegeneration induced by organ phosphorus compounds and chemical warfare agents.

PNPLA7 (Patatin Like Phospholipase Domain Containing 7): Human patatin-like phospholipases, such as PNPLA7, have been implicated in regulation of adipocyte differentiation and have been induced by metabolic stimuli.

PLA2G15 (Phospholipase A2 Group XV) is a lysosomal enzyme and has both calcium-independent phospholipase A2 and transacylase activities and catalyzes the formation of 1-O-acyl-N-acetylsphingosine and the concomitant release of a lyso-phospholipid. It is required for normal phospholipid degradation in alveolar and peritoneal macrophages and in spleen.

PLA2G4A (Phospholipase A2 Group IVA) is a member of the cytosolic phospholipase A2 group IV family and catalyzes the hydrolysis of membrane phospholipids to release arachidonic acid which is subsequently metabolized into eicosanoids. The enzyme is activated by increased intracellular Ca (2+) levels and phosphorylation, resulting in its translocation from the cytosol and nucleus to perinuclear membrane vesicles. Together with its lysophospholipid activity, it is implicated in the initiation of the inflammatory response.

PLA2G7 (Phospholipase A2 Group VII) is a secreted enzyme that catalyzes the degradation of platelet-activating factor to biologically inactive products. It modulates the action of platelet-activating factor (PAF) by hydrolyzing the sn-2 ester bond to yield the biologically inactive lyso-PAF. It is inactive against long-chain phospholipids.Platelet-activating factor acetylhydrolase deficiency (PAFAD).

ALOX12 (Arachidonate 12-Lipoxygenase) is a non-heme iron-containing dioxygenase that catalyzes the stereo-specific peroxidation of free and esterified polyunsaturated fatty acids generating a spectrum of bioactive lipid mediators. It has a dual activity since it also converts leukotriene A4/LTA4 into both the bioactive lipoxin A4/LXA4 and lipoxin B4/LXB4. Through the production of specific bioactive lipids like (12S)-HPETE it regulates different biological processes including platelet activation.

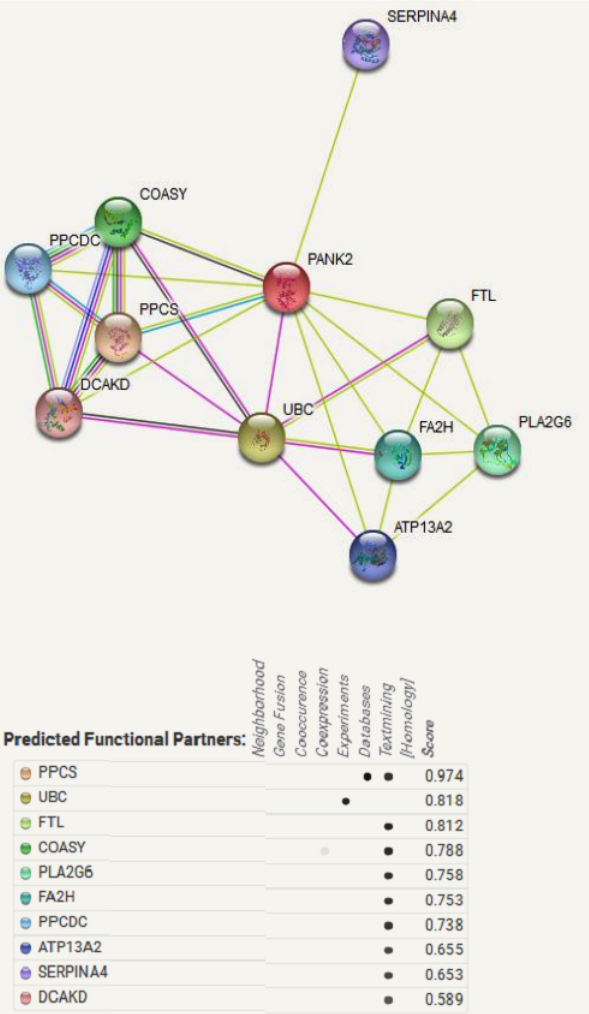
PLD2 (Phospholipase D2) may have a role in signal-induced cytoskeletal regulation and/or endocytosis and it catalyzes the hydrolysis of phosphatidylcholine to phosphatidic acid and choline. This protein localizes to the peripheral membrane and may be involved in cytoskeletal organization, cell cycle control, transcriptional regulation, and/or regulated secretion. Diseases associated with PLD2 include tooth agenesis.

LPCAT2 (Lysophosphatidylcholine Acyltransferase 2) possesses both acyltransferase and acetyltransferase activities, which is calcium-dependent, and it is involved in platelet-activating factor (PAF) biosynthesis. Upon acute inflammatory stimulus, acetyltransferase activity is enhanced and PAF synthesis increases. This protein is involved in the pathway phospholipid metabolism, which is part of Lipid metabolism.

PLA2G2A (Phospholipase A2 Group IIA) is a member of the phospholipase A2 family (PLA2) which catalyzes the hydrolysis of the sn-2 fatty acid acyl ester bond of phosphoglycerides, releasing free fatty acids and lysophospholipids, and thought to participate in the regulation of the phospholipid metabolism in biomembranes. It is a potent mediator of the inflammatory process, enhanced by inflammatory cytokines as well as lipolysaccharides in various cell types, elevated in numerous inflammatory diseases including sepsis, Crohn disease and pancreatitis, deleted in neuroblastoma.

PTGS2 (Prostaglandin-Endoperoxide Synthase 2) is the key enzyme in prostaglandin biosynthesis, and acts both as a dioxygenase and as a peroxidase. It is regulated by specific stimulatory events, suggesting that it is responsible for the prostanoid biosynthesis involved in inflammation and mitogenesis. This protein constitutively expressed in some tissues in physiological conditions, such as the endothelium, kidney and brain, and in pathological conditions, such as in cancer. PTGS2 is responsible for production of inflammatory prostaglandins. Up-regulation of PTGS2 is also associated with increased cell adhesion, phenotypic changes, resistance to apoptosis and tumor angiogenesis.

Fig. 4 Possible interactions between PLA2G6 and other proteins using STRING software. It showes that these interactions may involve in diffirent features of NBIA diseases.



PLA2G6 is an A2 phospholipase which may play a role in phospholipid remodelling, arachidonic acid release, leukotriene and prostaglandin synthesis, fas-mediated apoptosis, and transmembrane ion flux in glucose-stimulated B-cells.

PPCS (Phosphopantothenoylcysteine Synthetase) catalyzes the first step in the biosynthesis of coenzyme A from vitamin B5, where cysteine is conjugated to 4-phosphopantothenate to form 4-phosphopantothenoylcysteine.

UBC (Ubiquitin C) is a polyubiquitin precursor. Ubiquitination has been associated with protein degradation, DNA repair, cell cycle regulation, kinase modification, endocytosis, and regulation of other cell signaling pathways.

FTL (Ferritin, Light Polypeptide) is the light subunit of the ferritin protein which is the major intracellular iron storage protein in prokaryotes and eukaryotes. It is composed of 24 subunits of the heavy and light ferritin chains and variation in ferritin subunit composition may affect the rates of iron uptake and release in different tissues. A major function of ferritin is the storage of iron in a soluble and nontoxic state. Defects in this light chain ferritin gene are associated with neurodegeneration with brain iron accumulation 3 and hyperferritinemia-cataract syndrome.

COASY (Coenzyme A Synthase) functions as a carrier of acetyl and acyl groups in cells and thus plays an important role in numerous synthetic and degradative metabolic pathways in all organisms. In eukaryotes, CoA and its derivatives are also involved in membrane trafficking and signal transduction. The bifunctional protein coenzyme A synthase (CoAsy) carries out the last two steps in the biosynthesis of CoA from pantothenic acid (vitamin B5). Diseases associated with COASY include neurodegeneration with brain iron accumulation 6 and coasy protein-associated neurodegeneration.

FA2H (Fatty Acid 2-Hydroxylase) catalyzes the synthesis of 2-hydroxysphingolipids, a subset of sphingolipids that contain 2-hydroxy fatty acids. Sphingolipids play roles in many cellular processes and their structural diversity arises from modification of the hydrophobic ceramide moiety, such as by 2-hydroxylation of the N-acyl chain, and the existence of many different head groups. Diseases associated with FA2H include leukodystrophy dysmyelinating with spastic paraparesis with or without dystonia.

PPCDC (Phosphopantothenoylcysteine Decarboxylase) is involved in biosynthesis of coenzyme A (CoA) from pantothenic acid (vitamin B5) which is an essential universal pathway in prokaryotes and eukaryotes. PPCDC (EC 4.1.1.36), one of the last enzymes in this pathway, converts phosphopantothenoylcysteine to 4-prime-phosphopantetheine.

ATP13A2 (ATPase 13A2) is a member of the P5 subfamily of ATPases which transports inorganic cations as well as other substrates. May play a role in intracellular cation homeostasis and the maintenance of neuronal integrity.Mutations in this gene are associated with Kufor-Rakeb syndrome (KRS), also referred to as Parkinson disease 9.

SERPINA4 (Serpin Family A Member 4) inhibits human amidolytic and kininogenase activities of tissue kallikrein by formation of an equimolar, heat- and SDS-stable complex between the inhibitor and the enzyme, and generation of a small C-terminal fragment of the inhibitor due to cleavage at the reactive site by tissue kallikrein

DCAKD (Dephospho-CoA Kinase Domain Containing)

Fig. 5 Possible interactions between PANK2 and other proteins using STRING software. It reveals that these possible associations may involve in different characteristics of NBIA disorders.